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Non-typeable *Haemophilus influenzae* chronic colonization in chronic obstructive pulmonary disease (COPD)

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ABSTRACT

Haemophilus influenzae is the most common cause of bacterial infection in the lungs of chronic obstructive pulmonary disease (COPD) patients and contributes to episodes of acute exacerbation which are associated with increased hospitalization and mortality. Due to the ability of *H. influenzae* to adhere to host epithelial cells, initial colonization of the lower airways can progress to a persistent infection and biofilm formation. This is characterized by changes in bacterial behaviour such as reduced cellular metabolism and the production of an obstructive extracellular matrix (ECM). Herein we discuss the multiple mechanisms by which *H. influenzae* contributes to the pathogenesis of COPD. In particular, mechanisms that facilitate bacterial adherence to host airway epithelial cells, biofilm formation, and microbial persistence through immune system evasion and antibiotic tolerance will be discussed.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive but preventable condition associated with loss of lung function due to prolonged exposure to noxious particles or gases (Vogelmeier et al. 2017). It is typically characterized by airway obstruction as a consequence of small airway disease and parenchymal destruction in varying proportions (Rabe and Watz 2017).

An increase in disease severity is linked to a greater frequency of events associated with a sudden decline in lung function, known as an acute exacerbation of COPD (AECOPD) (Vogelmeier et al. 2017). These heterogeneous events, caused by interactions between pathogens, the lung environment, and the associated host response, lead to an increase in airway inflammation and a worsening of clinical symptoms (Sapey and Stockley 2006; Sethi and Murphy 2008). The disrupted innate lung defences observed in patients with COPD contribute to an increased frequency of airway infections making the patient more susceptible to AECOPD

(Sethi 2010). Knowledge of the nature of the inflammatory changes within the airways during exacerbations is, however, limited due to the relative risks involved in carrying out bronchial biopsies in patients with moderate or severe COPD (Hattotuwa et al. 2002).

Viruses are detected in approximately 50% of COPD exacerbations (Seemungal et al. 2001; Rohde et al. 2003), but an association between exacerbations and bacterial infections is more difficult to assess. This is because, in addition to acute bacterial infections driving the acquisition of AECOPD, the airways of COPD patients are intermittently colonized by pathogenic bacteria, with bacteria present at both stable and periods of exacerbation. Molecular science techniques, such as strain typing, allow specific bacterial strains to be isolated from patients and have provided evidence that the acquisition of new bacterial strains is a key trigger for an exacerbation (Sethi et al. 2002; Veeramachaneni and Sethi 2006). The associated, inappropriately elevated, inflammatory response (Sethi and Murphy 2008) is then driven by the higher levels of immune cells

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present in COPD airways such as macrophages, neutrophils, eosinophils, and dendritic cells (Di Stefano et al. 1994; Van Pottelberge et al. 2010; Barnes 2014; Finney et al. 2014).

Of all the bacterial strains capable of colonizing the respiratory tract of COPD patients, *Haemophilus influenzae* is arguably the most clinically relevant. Non-typeable *H. influenzae* (NTHi) is frequently isolated from COPD patients (Sethi et al. 2002) and is associated with up to 30% of acute exacerbations (Sethi 2010). Additionally, NTHi is detected in ~30% of COPD patients during stable disease over extended periods of time (Patel et al. 2002; Wilkinson et al. 2003; Marin et al. 2010). This indicates that NTHi utilizes a number of mechanisms in the airways that surmount innate and adaptive host defences as well as antibiotic treatments. This review will summarise the current understanding of *H. influenzae* biofilm formation and other mechanisms employed to enable its persistence within the COPD lung environment.

Haemophilus influenzae

H. influenzae is a human-restricted, Gram-negative coccobacillus that exists as a commensal organism within the nasopharyngeal flora of most humans (Erwin and Smith 2007). When growing aerobically, *H. influenzae* requires the presence of haemin and nicotinamide adenine dinucleotide (NAD; factors X and V, respectively) (Artman et al. 1983). Strains of *H. influenzae* are classified based on the presence, or absence of a chemically distinct polysaccharide capsule, which is divided into six serotypes (a–f) (Falla et al. 1994). Strains lacking a polysaccharide capsule are designated as NTHi.

As well as being a major cause of AECOPD, *H. influenzae* has been implicated in other conditions such as the inflammatory disease of the middle ear, otitis media (OM), and pneumonia (Gilsdorf et al. 2004). *H. influenzae* normally spread from person to person by inhalation of respiratory droplets or through direct contact of respiratory secretions (King 2012). *H. influenzae* serotype b (Hib) strains cause invasive infections in infants and children but since the development of the Hib conjugate vaccine, infection by *H. influenzae* is predominately due to NTHi (Van Eldere et al. 2014; Zarei et al. 2016). NTHi strains commonly colonize the upper respiratory tract and cause mucosal infections in children and adults (Agrawal and Murphy 2011).

Initial adherence of NTHi to the COPD airway

The first step in the timeline of bacterial infection is the initial adhesion to the host. As a respiratory pathogen,

NTHi must, however, first overcome the primary innate defences of the mucociliary escalator before attachment to the airway epithelium can occur (Ganesan et al. 2013). Effective mucociliary clearance (MCC) is dependent on the regulation of an optimal airways surface liquid by apical plasma membrane ion channels, ciliary beating, and appropriate levels of mucin secretion (Figure 1(A)); however, these can all be dysregulated in COPD (Figure 1(B)) (Ghosh et al. 2015). Cigarette smoke exposure, a common cause of COPD, decreases the activity of the cystic fibrosis transmembrane conductance regulator (CFTR; a cAMP-dependent chloride channel), which leads to airways dehydration (Clunes et al. 2012). Cigarette smoke exposure is also associated with a decrease in ciliary beat frequency (Yaghi et al. 2012), with lipooligosaccharide present in the cell wall of NTHi and protein D shown to further inhibit ciliary function (Denny 1974; Johnson and Inzana 1986; Janson et al. 1999). Airway mucus hypersecretion is also recognized as a key physiological feature of COPD (Rogers and Barnes 2006). This combination of thick, dehydrated mucous secretions and a decreased ciliary beat frequency severely disrupts MCC facilitating colonization of the airways by bacteria such as NTHi (King 2012) (Figure 1). NTHi subsequently uses a number of specialised cell surface proteins (summarised in Figure 2) to adhere to and invade the respiratory mucosa, enabling escape from host cellular immune defences.

The type IV pili (T4P), regulated by the products of the *pilA* and *comE* genes, are composed of helically arranged pilin subunits that form filamentous polymer assemblies. Their presence on the surface of a number of Gram-negative bacteria facilitates the uptake of DNA across the bacterial cell membrane, mediates adherence to mammalian epithelial cells and promotes genetic adaptability which also contributes to biofilm formation (Jurcisek et al. 2007). T4P exhibits high levels of adherence to the Intracellular Adhesion Molecule-1 (ICAM-1) which promotes epithelial cell adherence (Figure 2(A)). ICAM-1 is an adhesion molecule that is part of the immunoglobulin superfamily. It facilitates reversible adhesion and signal transduction between cells but in airways, cells are also exploited by a number of bacterial and viral pathogens such as NTHi and rhinovirus to enable their own adhesion and uptake.

ICAM-1 is present on the surface of a number of different cell types including airway epithelial cells (AECs) at a basal level but is increased following exposure to cigarette smoke (Scott and Palmer 2003). Increased levels of ICAM-1 are also observed in AECs of smokers and COPD patients (Shukla et al. 2017). Exposure to NTHi

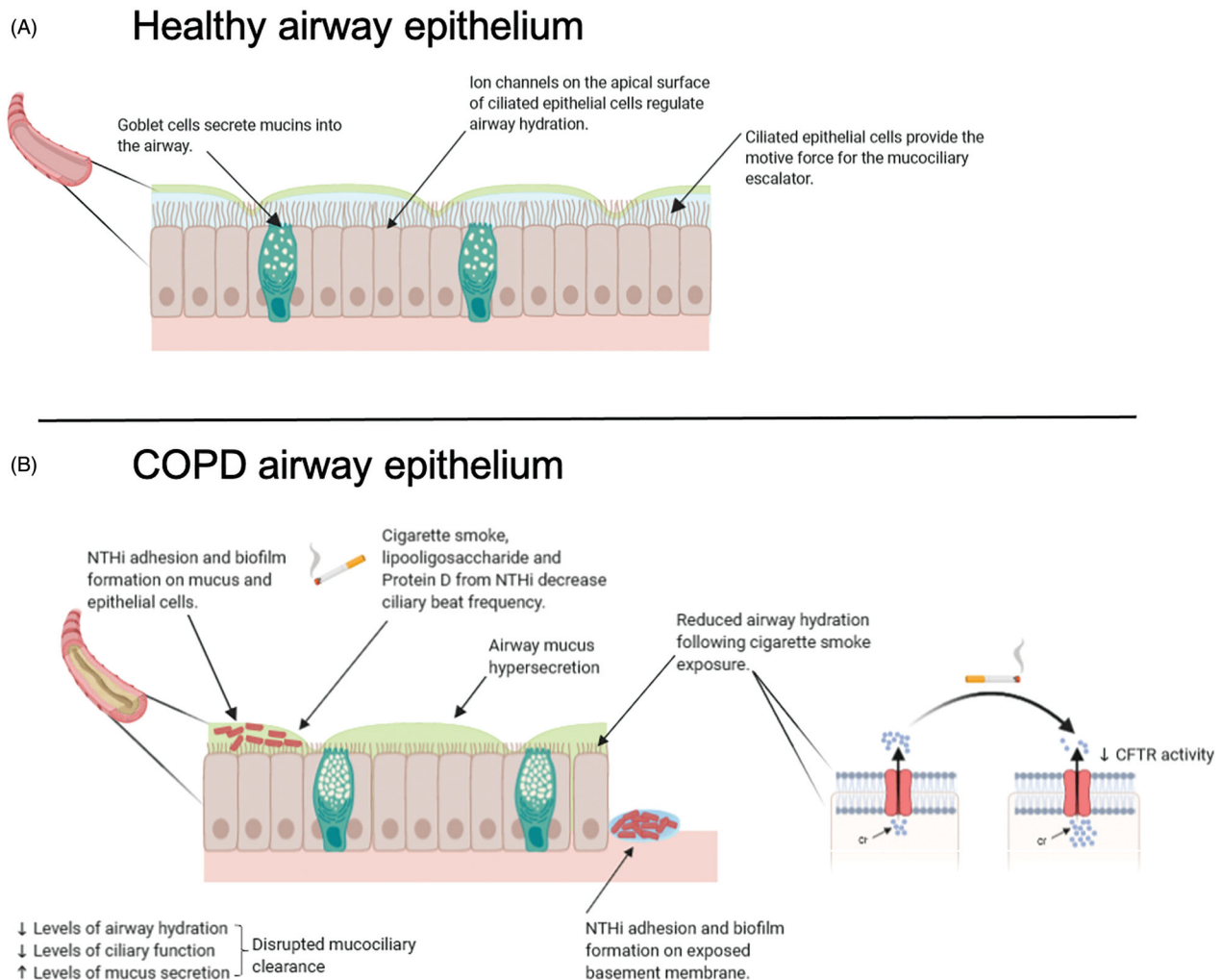


Figure 1. Summary of pathophysiological changes to the airway epithelium in COPD patients leading to an increased frequency of infections. (A) Healthy airway epithelium showing optimal airway hydration and effective mucociliary clearance. (B) COPD airway epithelium showing a reduction in the level of airway hydration through decreased Cl^- release by cystic fibrosis transmembrane conductance regulator (CFTR) ion channels, present on the apical surface of ciliated airway epithelial cells. Exposure to cigarette smoke and lipooligosaccharide and protein D from non-typeable *Haemophilus influenzae* (NTHi) lead to a decrease in ciliary beat frequency. Mucus hypersecretion is also observed in COPD airways. These changes lead to the accumulation of thick mucus that is not easily expelled from the airway, disrupting mucociliary clearance and facilitating initial bacterial adherence to the airway. Exposed sections of the basement membrane also facilitate adhesion of NTHi to extracellular matrix components present in the basement membrane. Created with BioRender.com.

can increase the expression of its receptor in AECs through upregulation of ICAM-1 (Avadhanula et al. 2006). The presence of further attachment points on the surface of AECs then act as a positive feedback loop for attachment and infection by NTHi. The P1 fimbriae bind to the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) cell surface glycoprotein present on the surface of epithelial cells (Tchoupa et al. 2015), whereas the P5 fimbriae facilitate cellular adhesion via mucins, CEACAM-1 and ICAM-1 (Avadhanula et al. 2006). Additionally, the NTHi high molecular weight adhesins Hmw1 and Hmw2 are key glycoproteins that display high levels of homology and are also key proteins in the process of binding to respiratory

epithelial cells (Geme and Yeo 2009; Rempe et al. 2016). Expression of both Hmw1 and Hmw2 are associated with invasive and virulent strains of *H. influenzae* (Vuong et al. 2013). In addition to Hmw proteins, *H. influenzae* possesses proteins that mediate adhesion to epithelial cells. Using dual RNA-sequencing, Baddal et al. (2015) identified the upregulation of multiple virulence factors, which included the gene encoding *H. influenzae* adhesion (Hia), another cell surface adhesion molecule found to mediate adherence to AECs (Spahich and St Geme 2011).

NTHi also utilizes the exposed extracellular matrix (ECM) present within the COPD airway environment to facilitate attachment by *Haemophilus* adhesion protein

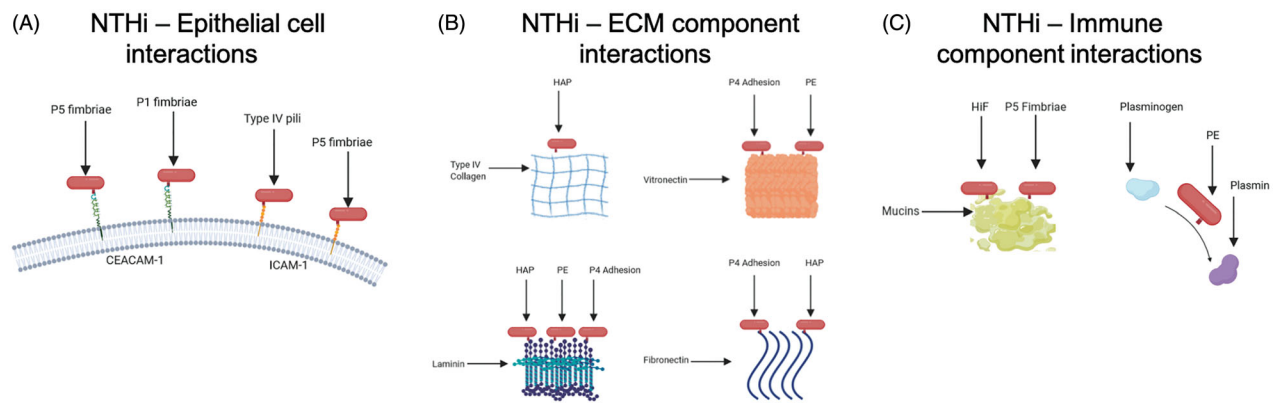


Figure 2. Summary of the roles of adhesion molecules present in strains of non-typeable *Haemophilus influenzae* (NTHi). (A) Interactions of P1 and P5 adhesins as well as the type IV pili with cell surface glycoproteins CEACAM-1 and ICAM-1 to facilitate adherence to the surface of airway epithelial cells; (B) highlights the roles of the HAP, P4 adhesin and PE in the adherence of NTHi to components of the basement membrane present in the airways, and (C) shows the adhesion of HiF and the P5 fimbriae to mucins present in the mucus secreted in the airway as well as the enzymatic conversion of plasminogen to plasmin by PE to disrupt the complement cascade. Created with BioRender.com.

(HAP) (Fink et al. 2002; Houghton et al. 2006) (Figure 2(B)). HAP is a ubiquitously expressed adhesion protein that binds to basement membrane components such as fibronectin, laminin, and collagen IV (Fink et al. 2002; De Chiara et al. 2014; Sekiguchi and Yamada 2018). HAP, however, is not required for biofilm formation (Hendrixson and St Geme 1998). Additionally, the P4 adhesion fimbriae adheres to fibronectin, laminin, and vitronectin and, as with P1, P2, and P5, are present in biofilms (Murphy and Kirkham 2002; Wu et al. 2014a; Su et al. 2016). Protein E (PE) of NTHi is involved in adhesion and activation of epithelial cells (Singh et al. 2010), interacts with laminin and vitronectin present within the ECM (Hallstrom et al. 2011), and upregulates IL-8 and ICAM-1 expression in AECs (Singh et al. 2013).

H. influenzae fimbriae (HiF) bind to mucins (Kubiet et al. 2000). In COPD mucus hypersecretion and defective MCC provide a relatively static mucin-rich environment which facilitates the adherence of HiF (along with the P5 Fimbriae) (Figure 2(C)). PE is also involved in evading host defences by binding to the proenzyme plasminogen causing its catalytic conversion to plasmin (Osman et al. 2018), a serine protease which, in addition to its primary role in fibrinolysis, has been shown to inhibit the complement pathway. The adhesion molecules described above, facilitate initial adhesion to AECs and also highlight the adaptations that allow HTHi to exploit the damaged, chronically inflamed environment present in the airways of COPD patients. Although adhesion to the airways facilitates infection, it is also the critical first step in the formation of a biofilm (Rabin et al. 2015).

***NTHi* biofilms and bacterial persistence**

A widely utilized mechanism of bacterial persistence within the respiratory tract is the formation of biofilms (Murphy and Kirkham 2002; Murphy et al. 2004; 2005). Biofilms are described as a community of cells encased in a self-produced ECM, adhered to a biotic or abiotic surface (López et al. 2010). In the airways, biofilms may act as reservoirs of bacteria, which can trigger the reoccurrence of lower respiratory infections and contribute to frequent exacerbator phenotypes in COPD (Sethi 2010). They are characterized by changes in cell behaviour, such as differential gene expression and ECM production which, in turn, can lead to increased virulence, antimicrobial tolerance and decreased susceptibility to killing from immune cells (Singh et al. 2017) (Figure 3). Biofilms also differ significantly in structure within the lungs. Instead of forming the “traditional” mushroom-like structure, they take on a small aggregate phenotype (Darch et al. 2018). These aggregates contain between 10^1 and 10^4 cells whilst retaining biofilm-associated characteristics such as increased antimicrobial tolerance.

***Haemophilus influenzae* biofilm formation**

Using a multi-omics approach, Harrison et al. (2019) characterized the transcriptome, metabolome, and proteome of *H. influenzae* to identify the driving forces behind its biofilm formation. Significant increases in amino acids and enzymes involved in central metabolic processes such as serine and glycine were identified, along with increases in adhesin production in combination with enriched DNA metabolism pathways.

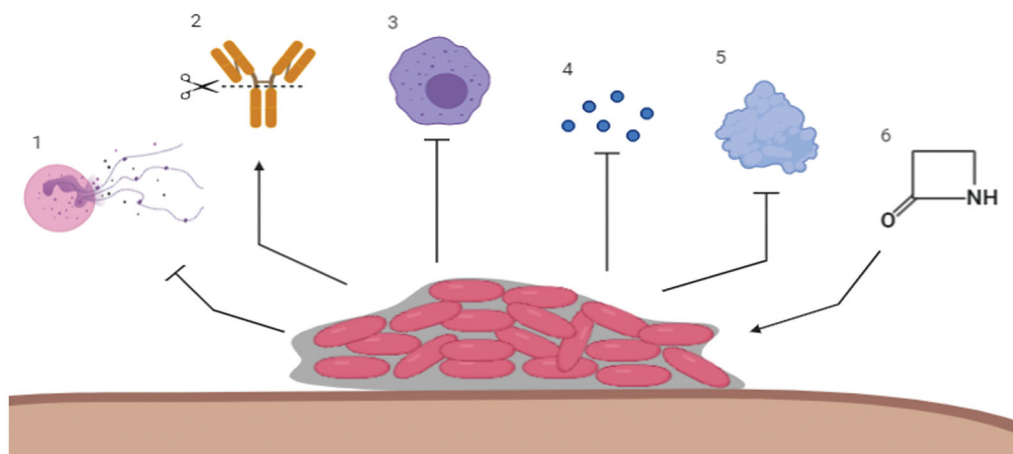


Figure 3. *Haemophilus influenzae* biofilms interact with the host and antibiotics. *H. influenzae* biofilms utilize a number of mechanisms to persist in the host. Production of a robust ECM aids in the resistance to neutrophils and NETS (1) and IgA proteases on the matrix surface cleave IgA (2). The thick ECM also prevents phagocytosis by blocking macrophage access to biofilm cells (3), pairing this with lowered cell metabolism and protein synthesis the activity of antibiotics (4) on biofilm cells is minimal. Another important mechanism of resistance is eDNA that is incorporated into the biofilm matrix that binds β -defensin (5), reducing its antimicrobial properties. Finally, likely as a stress response, β -lactam antibiotics induce biofilm formation in *H. influenzae* (6). Flat headed arrows indicate an inhibitory effect whereas pointed arrows indicate a stimulatory effect. Created with BioRender.com.

Another interesting observation from the same study was a significant increase in the expression of an uncharacterized ATP-binding cassette (ABC) periplasmic protein. Many members of this protein family provide bacteria with multi-drug resistance (Wilkins 2015) therefore, the increased expression of these pathways has been hypothesized to increase *H. influenzae* survival *in vivo* through contribution to horizontal gene transfer throughout the biofilms.

Biofilm communities have been studied *in vitro* for decades but it is extremely difficult to properly mimic *in vivo* conditions such as host immune responses and nutrient availability (Bjarnsholt et al. 2013). Biofilms formed by *H. influenzae* *in vivo* can, therefore, differ greatly from those formed *in vitro* (Brown et al. 2019b). From analyzing images of *in vivo* *H. influenzae* biofilms formed on the middle ear of chinchillas, Brown et al. (2019b) developed a reproducible, *in silico*, agent-based model to simulate biofilm formation *in vivo*. Results of this model showed that *H. influenzae* biofilms *in vivo* are formed following a similar spatial distribution pattern to their *in vitro* counterparts. However, *in vivo* biofilms were more than 10-fold smaller, likely as a result of host factors clearing planktonic and poorly integrated biofilm cells. These data highlight the importance of closely replicating host environments when studying biofilms in disease pathogenesis and provide a strong foothold to apply a similar approach to *H. influenzae* biofilms in the respiratory tract. Doing so

would be crucial in accurately studying *H. influenzae* biofilm pathogenesis in COPD.

Epithelial cell attachment

As previously discussed, adhesion proteins of *H. influenzae* play a key role in the attachment of the bacteria to epithelial cells which is the first step in the formation of biofilms. Indeed, it appears that *H. influenzae* increases adhesin protein production in response to contact with human respiratory epithelium as evident in co-cultures where the expression of T4P is significantly increased within 30 min following exposure (Mokrzan et al. 2019). In agreement with these findings, Baddal (2020) showed that *H. influenzae* adhesion to bronchial Calu-3 cells increased in a time-dependent manner, up to 72 h, at which point the colonizing bacterial cells were also found to induce epithelial cell apoptosis.

Quorum sensing

In many species of bacteria, proper biofilm development is dependent on the release and uptake of density-dependent small molecules, known as quorum sensing (Miller and Bassler 2001). In biofilms, quorum sensing pathways can regulate development, whereas others can induce or promote biofilm dispersal. One such quorum sensing network is the auto-inducer 2 (AI-2) pathway which is controlled by the *luxS* operon. Utilizing an *H. influenzae* mutant, where the *luxS* operon was controlled by the *xylA* promoter (which is activated

in the presence of xylose), Pang et al. (2018) showed that *luxS* operon activation and interruption influenced biofilm maturation and dispersal, respectively. Induction of *luxS* synthesis also resulted in increased transcription of a glycosyltransferase enzyme. In other bacterial biofilms formed by streptococcal species, which are also frequently identified in the lungs of COPD patients, glycosyltransferases are key enzymes involved in the production of biofilm ECM (Souza et al. 2020).

Extracellular DNA

Additional key components of *H. influenzae* biofilms released into the surrounding area, with multiple crucial roles, are extracellular DNA (eDNA) and DNABII proteins (Marti et al. 2017; Devaraj et al. 2018). *H. influenzae* $\Delta comE$ strains fail to secrete both proteins. *H. influenzae* T4P is also expressed through the pore formed by the *comE* product (Das et al. 2017; Jurgisek et al. 2017), suggesting this pilus acts as a type 4 secretion system that is utilized to “inject” eDNA and DNABII proteins into the biofilm matrix.

Biofilms and the immune system

The ECM of *H. influenzae* biofilms plays an integral role in resisting the effects of the host immune system by providing physical protection against phagocytosis and neutrophil extracellular traps (NETs) (Juneau et al. 2011; Langereis and Hermans 2013). In a study by Murphy and colleagues the formation of biofilms by clinical isolates of NTHi was found to be dependent on the production of a 30-kDa protein identified as peroxiredoxin–glutaredoxin (pdgX). PdgX levels were significantly increased in NTHi biofilms compared to planktonic growth and 44% of study participants, who were diagnosed with COPD, displayed considerably higher levels of antibodies to pdgX (Murphy et al. 2005). As well as being crucial for effective biofilm production, pdgX provides protection against oxidative stress-induced by neutrophils invading NETs (Juneau et al. 2015). The ability of *H. influenzae* biofilms to resist NETs, the production of which is already compromised in COPD (Pullan et al. 2015), provides ample opportunity for *H. influenzae* biofilms to persist within the airways for extended periods of time.

As well as the underlying transcriptional changes that accompany biofilm development, production of a robust ECM and immunoglobulin A (IgA) proteases are key to the immunoresistance and persistence showed by *H. influenzae*. IgA is a major component of the immune system and a first-line defence mechanism of mucosal surfaces such as the respiratory epithelium.

Increased levels of IgA have also been identified in COPD patients compared to healthy non-smokers (Ladjemi et al. 2015). IgA proteases produced within a biofilm can integrate into the ECM but are primarily found on the matrix surface where they thwart the host immune response by cleaving human IgA, a critical first line immunoglobulin localized in mucus membranes. The *H. influenzae* genome codes for two types of IgA proteases, *igaA* and *igaB*. All strains are able to produce *igaA* and 40% of strains have *igaB* (Murphy et al. 2015). There are two variants of each protease, with varying specificities of the hinge region found in human IgA. These proteases are variably expressed *in vivo* but play an important role in bacterial survival in respiratory epithelial cells (Murphy et al. 2017). Although this is yet to be confirmed, *igaB* appears to play a larger role in bacterial survival than *igaA*.

In addition to stimulating biofilm formation, eDNA binds to human β -defensin-3 (BD3), reducing its antimicrobial properties (Jones et al. 2013). BD3 is a member of the innate immune system and is crucial in protecting respiratory epithelial cells from invading bacteria (Andresen et al. 2011). Therefore, eDNA production by *H. influenzae* biofilms is essential for minimizing the antimicrobial effects of BD3 which is upregulated in those with COPD compared to healthy individuals (Andresen et al. 2011).

Antibiotic tolerance

Antibiotics are a commonly used tool in the treatments of AECOPD, however, sub-inhibitory concentrations of β -lactams have been shown to induce *H. influenzae* biofilm formation through the formation of tightly packed biofilms of increased overall biomass, albeit containing a reduced number of viable bacteria (Wu et al. 2014b). This highlights the importance of carefully considering antimicrobial options during the treatment of AECOPD to achieve proper microbial clearance and avoid microbial stimulation. The semi-dormant state of *H. influenzae* biofilms display reduced metabolism and protein synthesis while still maintaining the ability to modulate responses to oxidative stress (Post et al. 2014). This altered cell activity exacerbates the additional challenge of limited drug diffusion through biofilm ECM components (Singh et al. 2017). Decreased protein synthesis also minimizes the effects of antibiotics that operate as protein synthesis inhibitors, such as macrolides (Kanol and Rubin 2010).

Despite a high level of heterogeneity between biofilms produced by clinical isolates of *H. influenzae* (Puig et al. 2014), biofilm size does not influence antimicrobial tolerance. Reimche et al. (2016) tested the effect of

4 antibiotics commonly used in the management of COPD (amoxicillin, azithromycin, clarithromycin, and ceftriaxone) on pre-formed *H. influenzae* biofilms and found no correlation between biofilms of differing biomass or thickness and their ability to survive treatment.

Heteroresistance is a phenomenon frequently observed in pathogenic bacteria where subgroups of bacteria possess a lower susceptibility to antibiotic treatment than the general population (Nicoloff et al. 2019). Planktonic *H. influenzae* have been shown to display a heteroresistant phenotype against imipenem (Cherkaoui et al. 2017). It can be expected that this tolerance would be strengthened within a biofilm community and likely attributable to the differential behaviour of biofilm integrated cells (Flemming et al. 2016). For example, fluctuating gene expression and cell metabolism can lead to the development of heteroresistant and persisting cell phenotypes.

Biofilms and cigarette smoke

In addition to β -lactams, cigarette smoke extract (CSE) and electronic cigarette vapour extract (ECVE) have been shown to significantly influence *H. influenzae* biofilm pathogenicity (Gilpin et al. 2019). The prevalence of smoking within COPD populations is well documented and prolonged tobacco use is associated with symptoms of progressive COPD (Liu et al. 2015). Therefore, it is also important to understand how CSE influences bacteria within the lungs. Due to its recent emergence as a trend in nicotine addiction, the long-term health impacts of electronic cigarettes remain enigmatic. For that reason, it is imperative that studies address the potential impact of ECVE on the COPD lung microbiome. Both CSE and ECVE significantly increased *H. influenzae* pathogenicity in the *Galleria mellonella* infection model while also causing a significant increase in interleukin-8 (IL-8) and tumour necrosis factor α (TNF- α). Although both extracts exhibited a stimulatory effect on the biofilm production of a number of isolates such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the effect on *H. influenzae* biofilm formation was not significant (Gilpin et al. 2019).

Polymicrobial biofilms

Biofilms are rarely composed of a single species and the clinical significance of these polymicrobial biofilms is becoming increasingly recognized. The first reports of the polymicrobial nature of biofilms can be dated back to over three centuries ago where Antoni van Leeuwenhoek drew illustrations representing *Streptococcus* chains and *Fusobacteria* which he had observed in dental plaque (Brown et al. 2019a). To date,

dental plaque remains one of the most studied and best-characterized models of polymicrobial biofilms. 16S RNA sequencing has shown that the oral microbiome is similar to the lung (Bassis et al. 2015), indicating a relationship between oral and lung microbiomes. Salivary flow and microaspiration have been identified as major causes of bacterial migration from the oral cavity to the lungs resulting in oral pathogens such as *Porphyromonas* and *Prevotella* entering the lower respiratory tract (Huffnagle et al. 2017). This migration creates an opportunity for *Porphyromonas gingivalis*, a prolific oral pathogen, to interact with other bacteria such as *Streptococcus*, one of the most abundant genera in COPD lungs (Zakharkina et al. 2013; Sinha et al. 2018; Millares et al. 2019); an interaction that is known to be synergistic, promoting biofilm formation (Simionato et al. 2006). Within a polymicrobial biofilm, organisms are in constant communication with one another either through direct contact or via the release and uptake of quorum sensing molecules (Peters et al. 2012).

As the lungs of COPD patients are home to a plethora of microorganisms (Garcia-Nuñez et al. 2015; Wang et al. 2016; 2017) multiple opportunities exist for pathogens such as *H. influenzae* to interact with other bacteria and fungi. Limited attention has, however, been given to characterizing the interactions between respiratory pathogens. A number of studies have focussed on the relationship between *H. influenzae* and *Streptococcus pneumoniae* and their impact on OM which show a competitive link between the organisms. For example, a study by Bair and Campagnari (2020) reports *S. pneumoniae* to cause a rapid decrease in viable *H. influenzae* cells in dual-species biofilms after 24 h, which supports findings by Thornton et al. (2011) who failed to observe both species together in the same specimen from children with OM.

In acute OM cases, when isolated in combination with *S. pneumoniae*, *H. influenzae* was significantly more likely to be able to produce biofilms (Vermee et al. 2019). These two pathogens have previously been reported to modulate the expression of each other's virulence genes with transcription of *H. influenzae* T4P significantly increased in dual-species biofilms (Cope et al. 2011). Using a chinchilla infection model of OM, a polymicrobial *in vitro* biofilm of both *H. influenzae* and *S. pneumoniae* was able to persist in the middle ear due to *H. influenzae* providing *S. pneumoniae* protection against amoxicillin treatment (Murrah et al. 2015). While previously, *H. influenzae* 86-028NP, a β -lactamase producing strain, was found to protect both planktonic and biofilm *S. pneumoniae* cells from amoxicillin

(Weimer et al. 2011). β -lactamase production is undoubtedly not the sole mechanism for antibiotic protection. Although the exact mechanisms behind this have yet to be explored, it could be hypothesised that the combined, increased production of bacterial ECM prevents diffusion of antimicrobials, therefore protecting both organisms.

As described above, *H. influenzae* has proven its ability to produce glucosyltransferases in a biofilm state (Pang et al. 2018). As shown previously by Souza et al. (2020), glucosyltransferases are essential, not just for ECM production but for the proper formation of multi-species biofilms by *Streptococcus oralis* and *Candida albicans*. As a large number of fungal genera have been identified in the COPD lung, future studies should include investigations into the mechanisms of interaction between *H. influenzae* and fungi as well as other respiratory bacterial pathogens.

Additional mechanisms of persistence

Epithelial cell invasion

NTHi utilizes a range of adhesin proteins to adhere to the human respiratory tract. If the host fails to clear the invading bacteria, NTHi cells can then follow one of two paths that ultimately result in chronic colonization. The first path leads to the bacterium being engulfed by host respiratory epithelial cells. Secondly, the bacteria may either remain on the epithelial cell surface or may be bound by other host factors, such as fibronectin, potentiating the development of a biofilm.

H. influenzae is widely regarded as an extracellular pathogen and has shown the ability to persist in the intercellular space between epithelial cells due to the adhesion and invasion protein Hmw1 (Mell et al. 2016). The ability of *H. influenzae* to cause the downregulation of protocadherin 8 and cadherin 6, which are important in epithelial cell–cell interactions, and claudin 3 and claudin 8, which play a role in tight junction formation, highlight other potential mechanisms that may explain the ability of *H. influenzae* to invade the intercellular space (Baddal et al. (2015). Downregulation of cytoskeleton and tight junction components of respiratory epithelial cells, the latter of which serve to seal the paracellular barrier, allows *H. influenzae* to easily and effectively penetrate into the intercellular space.

In addition to this, evidence also shows *H. influenzae* able to invade epithelial cells and persist. Ahearn et al. (2019) identified an open reading frame (ORF) in *H. influenzae* as a key virulence factor involved in cell invasion. Although the product of this ORF is yet to be fully characterized, the ability of *H. influenzae* cells lacking

ORF *NTHI1441* to invade bronchial epithelial cells was significantly reduced, even though cellular attachment was observed.

As mentioned above, *H. influenzae* also utilizes the P1 fimbriae, a receptor for bactericidal long-chain fatty acids (LCFA), to bind to epithelial cells via the CEACAM-1 receptor (Moleres et al. 2018). Once bound to CEACAM-1, *H. influenzae* can then invade the respiratory epithelium. *H. influenzae fadL* (which codes for the P1 fimbriae) null mutants are much less effective at cell invasion. Computational analyses of >450 *H. influenzae* isolate genomes showed that host factor pressure loss of function mutations in *fadL* was rare with the exception of strains acquired from lung infections, which ultimately reduces the invasion potential of *H. influenzae* but increases tolerance to bactericidal LCFA which may benefit long term persistence.

Genetic adaptations

Mechanisms such as antigenic variation, epigenetic variation, and phase variation are also exploited by *H. influenzae* to increase fitness and prolong persistence within the host (Ahearn et al. 2019). In addition, where encapsulated *H. influenzae* rely on their capsule to evade the immune system, NTHi strains have been forced to develop alternative mechanisms. A number of genes have been identified that are involved in protection against complement-mediated killing (Nakamura et al. 2011). The majority of these genes are related to lipooligosaccharide (LOS) synthesis. *Haemophilus influenzae* LOS structure is controlled by the expression of a number of genes such as *lic1A*, *lic2A*, *lic3A*, *lic3B*, and *lex2A* (Phillips et al. 2019). LOS gene expression is regulated via phase variation, which allows for *H. influenzae* to quickly adapt in response to the external environment and therefore increasing bacterial fitness while inhabiting the human respiratory tract. Expression of adhesion genes such as *hmw* and *hia* are also controlled through phase variation (Giufre et al. 2008; Attack et al. 2015b). For example, the passage of *H. influenzae* cells through a chinchilla model of infection selected for cells that highly expressed the Hia protein proving that *H. influenzae* rapidly responds to its environment.

Despite playing an integral role in colonization and persistence, surface adhesins such as Hmw1 and Hmw2 elicit a strong host immune response (Winter and Barenkamp 2014) but this response can be moderated by *H. influenzae* using phase variation. Davis et al. (2014) reported that *H. influenzae* is able to repress the expression of *hmw* genes due to the presence of 7 bp tandem repeats located within the promoter region.

This study also showed that the protein production decreases as the number of tandem repeats increase.

Epigenetic variation in *H. influenzae* is dependent on *modA* expression. *H. influenzae* possess over 20 variations of *modA* alleles (*modA1*–*modA22*). *ModA* genes encode a DNA-methyltransferase, which is prone to random on/off switching (Atack et al. 2015a). Clinical isolates of *H. influenzae* found in the nasopharynx or inner ear of healthy individuals and OM patients possessed high levels of variation in the expressed *modA* alleles (Atack et al. 2015a). A Δ *modA10* *H. influenzae*, which showed increased attachment to and invasion of human bronchiole epithelial cells, highlights further the importance of epigenetic variation in providing *H. influenzae* the necessary advantages to allow survival within the human respiratory tract (Vanwagoner et al. 2016). When “switched on” *modA2* increases bacterial susceptibility to oxidative stress and neutrophil-mediated killing (Brockman et al. 2017). Cells expressing *modA2* form biofilms with significantly greater biomass compared to when this gene is “switched off.” Despite greater biomass, the ECM of these biofilms was deficient in eDNA (Brockman et al. 2018). It can be deduced that in contrast to *modA10*, *modA2* is detrimental to the survival of *H. influenzae* as strains that possess *modA2* are unable to effectively produce ECM which has been identified as a major contributor towards biofilm recalcitrance to antimicrobial therapy and immune cell attack (Singh et al. 2017).

H. influenzae also exhibits heterogeneity between strains in the presence of surface antigens, the differential expression of which results in strain-specific immune responses (Sethi et al. 2004; De Chiara et al. 2014). Contrary to phase variation, antigenic variation in proteins such as P1, P2, and P5 occur as a result of insertion and deletion mutations which have been reviewed extensively elsewhere (Gilsdorf et al. 2004).

Future directions

Most of our understanding of *H. influenzae* biofilms and persistence during infection still comes from OM, highlighting a need to address the current gaps in our knowledge of COPD pathology. Future work in addressing the role of *H. influenzae* biofilms in COPD should focus on developing definitive criteria for the diagnosis of biofilms in the respiratory tract and methods that can be used to confirm their presence. For example, a promising new technique of real-time molecular imaging could prove useful in the identification of *H. influenzae* biofilms. This method uses the environmentally sensitive fluorophore, 7-nitrobenz-2-oxa-1,3-diazole

conjugated to polymyxin which fluoresces upon contact with the lipid A component of Gram-negative bacteria (Akram et al. 2018). More recent studies have shown that similar smart probes can be used to label cell metabolites (Benson et al. 2019). Combining these two methods may provide a crucial opportunity to develop a method of selectively labelling molecules associated with *H. influenzae* biofilms such as pgdX, which if successful, could allow for the rapid diagnosis of *H. influenzae* biofilms in the COPD lung or indeed other conditions.

Importantly, recent studies have begun to unravel the complex network of inter-kingdom interactions between bacteria and fungi in lung diseases such as cystic fibrosis and pneumonia. Despite the mycobiome of the COPD lung being previously defined (Su et al. 2015) the interactions between fungi and bacteria in COPD have yet to be studied in detail. Fungi such as *C. albicans* and *Aspergillus fumigatus* are known to regularly interact with bacteria causing more serious infection (Kean et al. 2017; Reece et al. 2017). Future studies should aim to confirm if there is indeed co-association of fungi and bacteria in the COPD lung and should investigate if these interactions play a role in the pathogenesis and progression of COPD.

Conclusions

As a pathogen with a major impact on the lives of COPD patients due to chronic colonization, it is important to understand the underlying mechanisms of persistence employed by *H. influenzae*. One of the many methods of persistence utilized by *H. influenzae* is the formation of biofilms. The development of these complex microbial communities provides numerous advantages that help evade the host immune system and present multiple challenges for effective antimicrobial therapies.

Despite current advances in the field of *H. influenzae* biofilms, some aspects of this process particularly around the perceived synergy of polymicrobial biofilms remain enigmatic. Future work should aim to build upon our understanding of *H. influenzae* biofilms within COPD airways which will be aided by the development of more refined and accurate diagnostic tools for *in situ* biofilm identification. The rapid and continuous advancement of sequencing tools should also be exploited to further characterize the molecular mechanisms behind *H. influenzae* biofilm formation. This would enable the identification of key metabolites and proteins within biofilm communities that could be utilized as novel targets for new antimicrobial therapies. The ability to prevent *H. influenzae* biofilm

development or the provision of a rapid diagnosis and effective treatment options would be an extremely beneficial step to reduce AECOPD and help stall the devastating progression of COPD.

Authors' contributions

All authors contributed to the writing and review of the manuscript.

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